Metabolic capabilities of Lactococcus lactis

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CHAPTER 1. GENERAL INTRODUCTION

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The bacterium *Lactococcus lactis*

Lactococcus lactis is a Gram-positive bacterium, commonly associated to dairy environments, which has originally been isolated from plants (Passerini et al., 2010; Song et al., 2017). There are four *L. lactis* subspecies: *L. lactis* subsp. *lactis, L. lactis* subsp. *cremoris, L. lactis* subsp. *hordniae*, and *L. lactis* subsp. *tructae* (Parapouli et al., 2013; McAuliffe, 2018). Importantly, *L. lactis* belongs to the lactic acid bacteria (LAB) family, and strains of the subspecies *lactis* and *cremoris* are used in the fermentation of food, to obtain cheese, yoghurt, sauerkraut and other products (Cavanagh et al., 2015).

The relevant role that *L. lactis* plays in the food industry is related to flavor formation and the production of lactic acid, which contributes to the preservation of the food products. In addition, the long history of research on this bacterium makes *L. lactis* a current model LAB in genetic engineering (McAuliffe, 2018). Thus, *L. lactis* is a successful example of application development, it has been used for the traditional manufacture of fermented products to its current use as a microbial cell factory (Morello et al., 2007).

The products from fermentation of sugars

The catabolism of sugars is a principal activity of LAB. The *L. lactis* metabolism converts sugars into high concentrations of pyruvate (Figure 1). Then, pyruvate is mainly metabolized to lactic acid (homofermentative pathway), but it can also give rise to ethanol and/or acetate (heterofermentative pathway) (Neves et al., 2005). Besides the resulting production of lactic acid, other fermentation end products are synthesized, and although their production occurs in low amounts, they contribute to desired product properties such as flavor and texture (Kleerebezem et al., 2000). For instance, the carbon-4 compounds

(C4) pathway leads to the production of the flavor compounds acetoin, diacetyl and 2-3-butanediol.

Besides sugars, some LAB are able to metabolize other substrates such as citrate. The citrate catabolism results in high production of carbon dioxide and the C4 compounds diacetyl, acetoin and 2,3-butanediol (Quintans et al., 2008). These C4 compounds are responsible for the aroma and flavor properties of dairy products. In particular, diacetyl is a highly desired product because it gives the appealing aroma of butter, margarine, sour cream, yogurt, and a number of cheeses, including Cheddar, Gouda, Camembert, Swiss, Maasdam, quarg, Mexican Chihuahua, ricotta, cottage, and goat cheeses (Clark and Winter, 2015). Strains of *Lactococcus lactis* subsp. *lactis* biovar diacetylactis, and some species of the *Leuconostoc* and *Weissella* species genera are used as diacetyl producers.

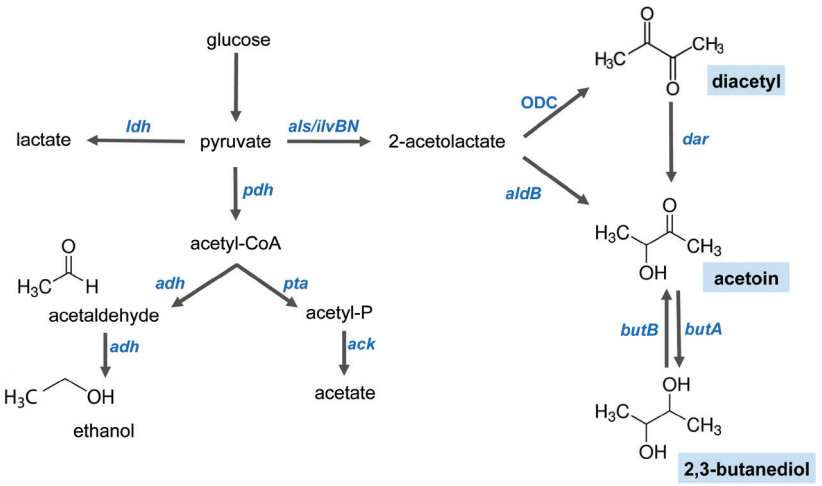

Figure 1. *L. lactis* fermentation products. After glucose is internalized in the cell, its breakdown results in pyruvate. The pyruvate molecules can be converted to several end products. Lactate is the main product of lactate dehydrogenase (*ldh*). Under aerobic conditions, pyruvate is decarboxylated by the pyruvate dehydrogenase (*pdh*) complex to produce acetyl-CoA. Acetaldehyde, ethanol and acetate are products of the activity of phosphotransacetylase (*pta*), aldehyde/alcohol dehydrogenases (*adh*) and acetate kinase (*ack*), respectively. Under aerobic and acidic conditions, a shift towards the 4-carbon compounds (indicated in blue boxes, diacetyl, acetoin and 2,3-butanediol) occurs. Diacetyl is produced by oxidative decarboxylation (ODC). Acetoin can be produced by activity of a 2-acetolactate dehydrogenase (*aldB*) or by diacetyl reduction by the diacetyl reductase (*dar*). 2,3-butanediol is produced by the acetoin dehydrogenase (*butA*), but this reaction is reversible and 2,3-butanediol can be converted into acetoin by the 2,3-butanediol dehydrogenase (*butB*).

Flavor formation by *L. lactis*

As mentioned above, the C4 compounds are minor end products of fermentations, but relevant for aroma and flavor. In addition to its natural appearance in dairy products, diacetyl has a high commercial value and it is manufactured for use as a food additive (Clark and Winter, 2015). Likewise, acetaldehyde is the major component of the yogurt flavor, which is a mixture of several compounds such as acetone, diacetyl and acetaldehyde (Chaves et al., 2002).

The pathway for diacetyl production by *L. lactis* subsp. *lactis* biovar diacetylactis has been extensively studied (Hugenholtz et al., 2000). The main route of diacetyl synthesis is via the intermediary compound α-acetolactate. The α-acetolactate synthase (Als) enzyme is responsible for the condensation of two pyruvate molecules to generate α-acetolactate. Once synthesized, α-acetolactate is unstable and is decarboxylated to acetoin by α-acetolactate decarboxylase (AldB), or by oxidative decarboxylation (in the presence of oxygen) to diacetyl. Acetoin can also be synthesized from diacetyl by diacetyl reductase (Dar). Then, 2,3-butanediol is produced from acetoin by the activity of acetoin reductase (ButA), a reverse reaction catalyzed by 2,3-butanediol dehydrogenase (ButB). These C4 compounds are secreted without requiring specific transporters.

Metabolic engineering strategies have attempted to increase the production of diacetyl by lactic acid bacteria. For instance, previous studies overproduced α-acetolactate synthase (Als) or inactivated lactate dehydrogenase (Ldh) (Benson et al., 1996; Kleerebezem et al., 2000). Although, these strategies have resulted in efficient conversion of lactose and glucose into acetoin, only low yields of diacetyl production were obtained.

Furthermore, flavor formation by *L. lactis* is derived from amino acid catabolism (Figure 2) (Kieronczyk et al., 2003). The amino acid catabolic pathways produce aldehydes, alcohols, carboxylic acids, and (thio)-esters (Yvon and Rijnen, 2001). There are two distinct routes for the conversion of amino acids to flavors, i.e. transamination and elimination. The branched chain amino acids, aromatic amino acids, and methionine are catabolized via the transamination route (Kieronczyk et al., 2004). This path is initiated by aminotransferases that convert amino acids into their corresponding α-keto acids. The α-keto acids are then further converted into aldehydes, alcohols, and esters, which are important aroma compounds. The elimination route has been described for methionine, where the carbon-sulfur lyases activity results in the release of methanethiol (Seefeldt and Weimer, 2000). The resulting compounds of the catabolism of amino acids play a role in flavor development of cheeses.

The final flavor of cheeses depends on the concentrations of different amino acids (Gutiérrez-Méndez et al., 2008). For instance, sensory analysis revealed that glutamate is the main source of umami taste in Cheddar and Swiss cheese, and the intensity of umami taste increases as more free glutamic acid is produced during cheese ripening (Yamaguchi and Ninomiya, 2000; Drake et al., 2007).

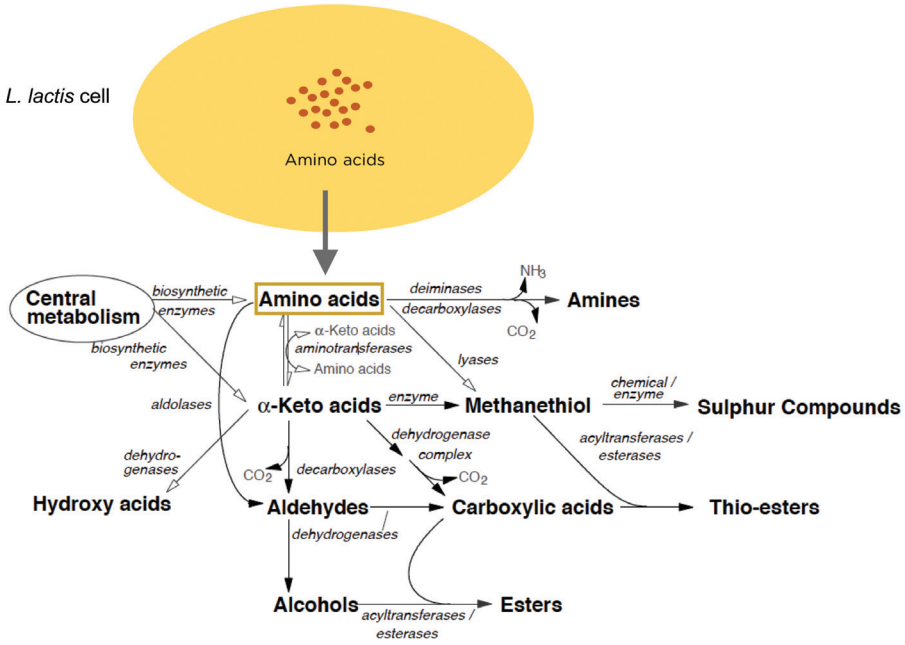

Figure 2. Amino acids are precursors of various flavor compounds. The amino acids inside of a *L. lactis* cell are subject to deaminases, decarboxylases, transaminases and lyases. The activity of all these enzymes results in aldehydes, alcohols, (thio)-esters or sulphur compounds with flavor properties. Based on van Kranenburg et al. (2002).

Nitrogen metabolism and the proteolytic system

Lactococci are fastidious organisms with regard to the medium in which they grow. Their growth requires several nutrients such as essential amino acids and vitamins (Aller et al., 2014). Although milk is a protein-rich medium, the amounts of essential amino acids it contains are very small. In fact, the limiting factor in cheese production is the low growth rate caused by the small amount of essential free amino acids in fresh milk (Thomas and Pritchard, 1987). For example, in spite of

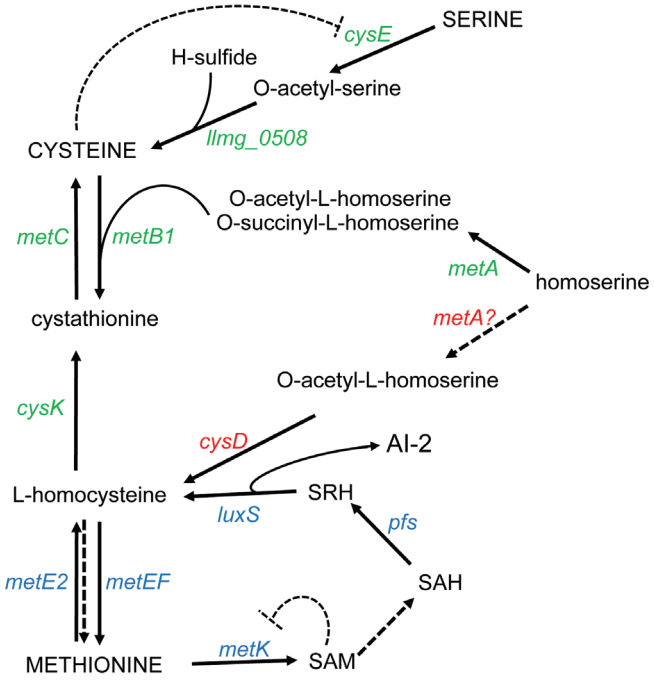

Figure 3. The methionine, cysteine and serine synthesis pathway in *L. lactis* MG1363. Methionine can potentially be synthesized using L-homocysteine as a substrate. The pool of homocysteine is derived from a recycling pathway via *metK, pfs* and *luxS*, or via interconversion of homoserine into L-homocysteine by *metA* and *cysD*. In a review of the literature, Sperandio et al, 2010 described the sulfur amino acid metabolism in the *L. lactis* IL1403 strain, and showed that cysteine might enter by an interconversion pathway to methionine. This conversion is not possible in *L. lactis* MG1363 due to the lack of the enzyme YtjE that converts cystathionine into homocysteine. Three transcriptional regulators participating in these biosynthetic pathways: genes regulated by CmbR in green, genes regulated by CodY in red, and genes that are potentially regulated by CmhR in blue.

the fact that plants and most microorganisms are able to synthetize methionine *de novo, L. lactis* MG1363 is auxotrophic for this amino (Figure 3). Methionine is an essential cellular compound due to its role as the universal N-terminal amino acid in protein synthesis and its participation in methylation reactions. In fact, the low methionine availability in milk is a limiting factor of growth of some lactic acid bacteria (Sperandio et al., 2005).

In milk, the concentration of essential amino acids (isoleucine, leucine, valine, histidine, glutamic acid and methionine) is less than 1 mg/L. This initial free

amino acids content in milk provides sufficient nitrogen for only 2% of the final cell density (Samaržija et al., 2001). Thus, as the proteins αs1- and β-casein are the major source of amino acids in milk, a good growth is dependent on a good proteolytic system that can liberate the amino acids, or precursors peptides, from casein (see Figure 4).

Caseins are the most abundant proteins in milk, and are the primary nitrogen source for lactococci. The casein proteolysis starts with the cell-envelope proteinase (CEP) named PrtP, a key enzyme of the proteolytic system which cleaves more than 40% of the peptide bonds into more than 100 different oligopeptides (Juillard et al., 1995). Not all *L. lactis* strains bear a functional PrtP, and these strains are mostly referred as nonstarter strains (PrtP-). These strains rely on starter strains (PrtP+) for the production of casein-derived peptides (Niven et al., 1998; Christensen et al., 1999). A functional proteinase PrtP requires the products of the *prtPM* genes. The *prtP* gene encodes the proteinase PrtP and it is located downstream the *prtM* gene, which encodes the PrtM enzyme responsible of autocatalytic maturation of PrtP (Haandrikman et al., 1989, 1991). Thus PrtM is essential to obtain a functional PrtP. Remarkably, the genes needed for growth in milk, i.e. for casein utilization, are encoded on large plasmids which are sensitive to rearrangements, loss and deletions (Kok, 1990). This observation has been explained by the fact that *L. lactis* obtained these plasmids during its adaptation to dairy environments (Passerini et al., 2010). Interestingly, the genetic susceptibility of the plasmids containing the *prtPM* genes, has resulted a variety of types of the proteinase PrtP (Juillard et al., 1995). In general two main PrtP types are distinguished, PI and PIII types, this classification is based on their substrate specificity for αs1-, β- and κ-caseins (Kunji et al., 1998; Børsting et al., 2015). PI-type primarily cleaves β-caseins into more than 100 different oligopeptides (form 4 to 30 residues), whereas PIII-type cleaves αs1-, β- and κ-caseins equally well. However, proteinases PrtP can be further classified into seven groups (a, b, c, d, e, f, g) based on specific amino acid residues of the PrtP protein sequence (Exterkate et al., 1993).

After the initial casein degradation, *L. lactis* utilizes several transport systems to take up the peptides derived from casein and created by the PrtP. The casein-derived peptides are imported to the cell via different transporter systems (see Figure 3). There are three peptide uptake systems: the oligopeptide transport system (Opp), the dipeptide/tripeptide system (DtpT) and the di- tri- and tetrapeptides system (Dpp) (Kunji et al., 1993; Sanz et al., 2001). The Opp system plays the biggest role in peptide internalization, because it mediates the uptake of pepti-

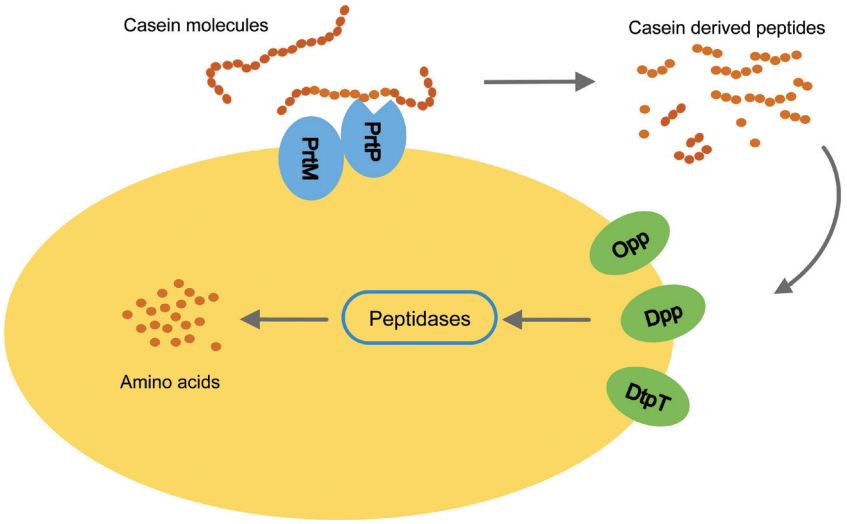

Figure 4. The *L. lactis* proteolytic system. Casein molecules present in milk are degraded by the proteinase PrtP. The cell via three different uptake systems (Opp, Dpp and DtpT) can take up the casein-derived peptides. Once the peptides are inside of the cell, they are further degraded by peptidases into free amino acids.

des with 4 to 18 residues. The Opp and Dpp systems are ATP-dependent and the DtpT system is a secondary transporter driven by a proton motive force (Hagting et al., 1994). Dpp transports a variety of different di- and tri-peptides, but preferentially the branched-chain amino acids (BCAAs), whilst DtpT transports the more hydrophilic and charged peptides (Kunji et al., 1995; Sanz et al., 2001). Moreover, It has been suggested that peptidases released into the medium after cell lysis, such as the intracellular endopeptidases PepO and PepF, could be responsible for di-tri-peptide production (den Hengst et al., 2005).

Next, the internalized peptides are further degraded by various internal peptidases into free amino acids (Savijoki et al., 2006). These amino acids can be used in several cellular processes such as protein synthesis, but also can be converted into other compounds such as the flavor compounds mentioned above. In a previous study with the *L. lactis* MG1363 strain (Lac+, Prt+), overproducing either PepN, PepC, PepO, or PepV, was used for pilot-scale Cheddar cheese trials, including two different trained sensoric panels that assessed the cheese organoleptic quality (Karimi et al., 2012). Bitter flavor was significantly reduced while flavor preference was increased only in those strains overproducing the general aminopeptidases PepN and PepC.

Remarkably, the breakdown products of

casein-derived peptides are either flavor compounds or flavor precursors, which undergo further conversion to the flavor compounds highly demanded by consumers. Additionally, there is a relationship between autolysis, proteolysis and flavor formation. High cell wall lytic activity has been correlated with high release of intracellular peptidase activity and with an increase in the amino nitrogen during cheese ripening (Chapot-Chartier et al., 1994; Crow et al., 1995). Important is also the observation that the proteinase PrtP degrades the *L. lactis* major autolysin AcmA (Buist et al., 1998). Thus, a fine balance is necessary between the cells with and without lysis.

Although *L. lactis* utilizes its proteolytic system to obtain essential amino acids to thrive in milk, amino acid transporters are also utilized to import the free amino acids available in milk. With this respect, several transporters have been described (Trip et al., 2013). For example, the ABC transporter GlnPQ that imports glutamate/glutamine (Schuurman-Wolters and Poolman, 2005) or the branched-chain amino acid transporters BcaP and BrnQ (Den Hengst et al., 2006; Trip et al., 2013).

Nitrogen metabolism- the genetic regulation

Studies on the proteinase PrtP have demonstrated that its production is inhibited by the presence of rich peptides in the growth medium. For instance, when *L. lactis* is grown in medium containing casitone, which is a tryptic digest of casein (Exterkate et al., 1993). The inhibition of the PrtP expression is observed also in chemically defined medium (CDM) supplemented with Leu-Pro (Laan et al., 1993). Accordingly, high PrtP expression has been reported in media containing low amounts of peptides, and the addition of Pro-Leu or Leu-Pro lowered the PrtP expression (Meijer et al., 1996). In addition, the *oppA* gene is repressed more than 20-fold when Leu-Pro or Pro-Leu are added to growth medium (Marreddy et al., 2010). The *oppA* gene encodes the oligopeptide transport system protein A, which is the enzyme responsible for binding to the peptides for transportation. Similarly, the endopeptidases PepX and PepN have low activity with increased concentration of casitone in whey-permeate medium (Meijer et al., 1996).

The signal controlling casitone-dependent repression of proteolysis is the intracellular pool of branched-chain amino acids (Guédon et al., 2001). The pleiotropic regulator CodY has been shown to repress *prtP* and *prtM* by its binding to the intergenic region between these genes. With this respect, previous studies demonstrated that *L. lactis* CodY directly interacts with the upstream region of the promoter of the *opp* operon encoding the oligopeptide transport system Opp (Den Hengst et al.,

2006). Moreover, the interaction of CodY with the target promoters is affected by the presence of branched-chain amino acids (Guédon et al., 2001).

Besides the regulation of the enzymes participating in the *L. lactis* proteolytic system by CodY, amino acid transporters are also subject to genetic regulation. The well-described branched chain amino acid permease (BcaP) is able to transport not only the branched-chain amino acids (BCAAs: isoleucine, leucine, valine), but also methionine, and to a lesser extent cysteine. CodY controls the expression of the branched-chain amino acid permease BcaP, where the *bcaP* promoter is repressed by the CodY regulator when BCAAs are abundant (Den Hengst et al., 2006). In addition, CodY also regulates the aminotransferases AraT and BcaT, which physiological role is to catalyze the last step in the biosynthesis of branched-chain or aromatic amino acids (Chambellon and Yvon, 2003).

Other important nitrogen regulators are the LysR-family regulators, MetR/MtaR, CmbR and HomR. In LAB or related Gram-positive bacteria, the control of transcription of sulfur amino acid metabolism is generally under control of these three LysR-family regulators (Liu et al., 2012). The *L. lactis* IL1403 strain lacks the CmbR regulator, but the CmhR regulator controls not only the cysteine uptake and biosynthesis, but also the methionine uptake via the expression of a methionine transporter encoded in the *plpABCD-ydcBCD* operon (Sperandio et al., 2005). Added to the role of the transcription factors such as CmhR, the Met-like transporters of *B. subtilis* and of most other Gram-positive bacteria are regulated by a S-box motif in the leader region of the *met* operon (Hullo et al., 2004).

Gram-positive bacteria have other nitrogen regulation systems involving premature transcription termination (T-box), which regulate the expression of various aminoacyl-tRNA synthetases and the genes involved in amino acid uptake and biosynthesis (Suddala et al., 2018). The T-box anti-termination mechanism is an elegant mechanism by which many bacteria control the amino acids levels in the cell (Henkin and Grundy, 2006). When there is sufficient charged tRNA in a cell, the T-box folds into a terminator structure, blocking transcription. In opposite, when there is uncharged tRNA in the cell, transcription proceeds upon the conversion of the T-box structure into an anti-terminator structure, which is induced by binding of a highly conserved 5'-NCCA-3' of the uncharged tRNA with a conserved '5-UGGN-3' sequence in the T-box sequence (Wels et al., 2008; Green et al., 2010). This regulation mechanism depends on the T-box specifier codon, which interacts with the anticodon of an uncharged tRNA. For example, the identi-

fication of His T-boxes in members of the *Lactobacillales*, was found upstream the genes related to ABC-type transporters. These operons are regulated by a T-box element with a histidine (CAC) specifier sequence, suggesting that the product is a His-transporter (Gutierrez-Preciado et al., 2009).

Amino acids

Amino acids are attractive metabolites in industrial microbiology. Besides their production as a bulk biochemical by fermentative procedures, they are relevant precursors of flavor compounds in dairy fermentations (Marin and Krämer, 2007; D'Este et al., 2018). These molecules can be organized in categories depending on their effect on *L. lactis* growth, essentiality, synthesis or influence on flavor formation (Table 1).

Since amino acids find application as flavoring agents, as feed additives, for pharmaceutical purposes or artificial sweeteners, the amino acids market demand has increased (Hirasawa and Shimizu, 2016; Pinu et al., 2018). New technologies towards the development of amino acid-producing microbial cells have been used. For example, the first described glutamate-secreting microorganism *Corynebacterium glutamicum* has been used in engineering approaches to increase the production of glutamate, lysine and other flavor active amino acids at a large-scale (Georgi et al., 2005; Zahoor et al., 2012).

Three fields where amino acids are relevant are the food-, pharmaceutical- and chemical industry. In food industry, amino acids can be used as flavor enhancers, for instance glycine and alanine enhance taste and flavor, and as an antioxidant, where cysteine is added to fruit juices (Solms, 1969; Yamaguchi and Ninomiya, 2000). In pharmaceutical chemistry, amino acids are components of several formulations, for instance histidine is added to antibody solutions because it has a protective effect against lyophilization-induced structural perturbations and increases the stability of the antibody during subsequent storage (Arakawa et al., 2007). In the chemical industry, amino acids have a great potential as sustainable and eco-friendly substrates for surfactant production such as the ones used in laundry detergents or emulsifiers (Tripathy et al., 2018).

Industrially, the secretion of amino acids by some microorganisms has an economic relevance for biotechnological fermentation procedures, as it simplifies the extraction and purification of these compounds (Krämer, 1994). The microbial production of amino acids is performed with enzymatic method or via fermentations (Ikeda, 2003). Firstly, in the enzymatic process one or more enzymes catalyze the production of the desired amino acids. Enzymes such as

Table 1. Relevant properties of amino acids in *L. lactis*. Based on (van Niel and Hahn-Hägerdal, 1999; van Kranenburg et al., 2002; Smit et al., 2009; Flahaut et al., 2013).

Essential (cannot be synthetized by *L. lactis*)
Obtained by degradation of β-casein
Glutamate
Glutamine
Methionine
Valine
Leucine/Isoleucine
Obtained by degradation of κ-casein
Histidine

Non-essential (synthesized from existing aa or from alpha-cetocarboxilates)
Arginine
Aspartate
Cysteine
Threonine
Tryptophan
Tyrosine

Growth stimulating amino acids
Asparagine
Proline
Phenylalanine
Alanine

Flavor promoting amino acids
Alcohol, aldehydes and acids
Valine
Leucine
Isoleucine
Sulphur aroma
Methionine
Cysteine
Floral/fruity notes
Tyrosine
Tryptophan
Phenylalanine

NAD^+-dependent L-amino acid dehydrogenases are used in these processes (Li et al., 2012). The main advantages of the enzymatic methods are the production of optically pure D- and L-amino acids at high concentrations, while low amounts of by-products are obtained. Secondly, in the fermentation process, microorganisms are used to convert sugars into amino acids. In this process, the main advantage is the production of only L-amino acids, and thus it does not require extra purification steps (Wendisch, 2014). The utilization of either the enzymatic or fermentation process depends on the existing technology for the desired amino acid or the operation costs related (Bongaerts et al., 2001).

Biologically, amino acids that are secreted by microorganisms may indicate overabundance of nitrogen resources. In the so-called overflow metabolism, nutrients are secreted in response to imbalanced metabolic pathways (Ponomarova et al., 2017). However, this argument is not valid for metabolites that

are secreted without intracellular accumulation, which suggests that another, yet unknown, strategy drives the secretion of metabolites (Pinu et al., 2018). Some mechanisms of amino acid secretion by bacteria have been described. Proline secretion by *Escherichia coli* or *Bacillus subtilis* occurs by both passive lipoidal (through bilayer) and via carrier-mediated diffusion (Nikaido, 1993; Lepore et al., 2011). Specific transporters for amino acids are also well described, for instance the secretion mechanism for L-glutamate in *C. glutamicum* occurs via small-conductance mechanosensitive channels (Mitsuashi, 2014). Interestingly, *Saccharomyces cerevisiae* is able to secrete amino acids via vesicles; the amino acids are loaded in intracellular vesicles, and then the vesicles are merged to the cytoplasmic membrane, which results in the release of the amino acids into the extracellular environment (Velasco et al., 2004).

Biosensors

Microorganisms produce extracellular compounds such as flavors, amino acids, antimicrobials and enzymes that contribute to the quality of final products of fermentation processes (Lim et al., 2015; van Tatenhove-Pel et al., 2020). However, some limitations to increase the production of a desired compound are that the compounds can be produced in low amounts, or there is the requirement of laborious methods for their quantification (Mahr and Frunzke, 2016). Moreover, the strain-engineering methods and the screening of wild-type strains with increased production levels are also laborious tasks. Biosensors can be utilized as a semi-quantitative tool to facilitate the detection and quantification of these compounds in complex food matrices (Thakur and Ragavan, 2013). With regard to the selection of overproducing mutants, the coupling of the extracellular product to the producer genotype can be achieved by single-cell compartmentalization.

Whole-cell biosensors are analytical tools that can be used for detection of a wide range of substances. These devices are an emerging technology in food industry as an alternative for the conventional analytical techniques. Cell-based sensors are advantageous in cost since a high number of cells can be obtained in low-cost nutrient media, whereas other sensor systems e.g. enzyme-based sensors require tedious purification processes and the use of analytical instruments such as chromatography, spectrophotometry or other techniques (Thakur and Ragavan, 2013; Lim et al., 2015). Recent engineering strategies provide microorganisms the desired properties as biosensors: increase in the dynamic range of a reporter output, enhance the sensitivity of a sensor construct, development of specificity to different effector molecules, and transfer of

sensing elements between organisms (i.e. orthogonality) (Mahr and Frunzke, 2016).

The concept of compartmentalization in microdroplets makes microbial cells suitable sensors for detection of secreted target molecules in these individual contained compartments. Previous research has established that the microscopic compartments reduce the time required to detect the molecules released by the cells. Moreover, since fluorescence measurements offer the most successful method to analyze millions of droplets in short time (Theberge et al., 2010), engineered biosensors have been developed to transduce the concentration of a

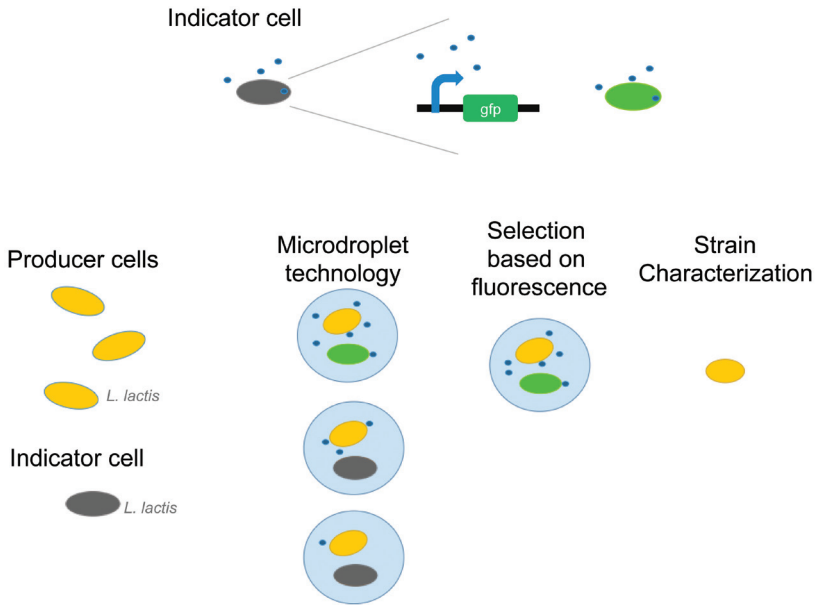

Figure 5. Selection of *L. lactis* overproducers: coupling biosensors with metabolite production via compartmentalization. An indicator cell (biosensor; in grey) consists of a compound-responsive promoter that drives the expression of the green fluorescent protein (GFP), i.e. the presence of a compound of interest (blue dots) is translated to green fluorescent cells. The co-cultivation of indicator cells and producer cells in micro compartments (microdroplet technology) allow the selection of individual producer cells with increased production of the compound of interest. Droplets with high concentrations of the compound show higher fluorescence levels compared to droplets with low concentrations. Further selection of the droplets and recovery of the producer cells is based on florescence with a high-throughput technique such as fluorescence assisted cell sorting (FACS). The compound production by the selected strains (characterization) confirms the selection procedure.

desired product into a fluorescent signal (see Figure 5).

The measurement of a secreted product via fluorescence can be achieved by three microbial-cell sensing systems: a cell bearing a sensing element coupled to a reporter gene, a constitutively fluorescent cell the growth of which depends on the product of interest, and the use of fluorogenic molecules for instance to detect growth inhibition of a sensor strain when the producer strain releases molecules with an inhibitory effect (Figure 6).

In the first group of microbial-cell sensors (Fig. 6A), besides the transcription-dependent sensors based on promoter activation/repression in response to the presence/absence of a molecule, new sensing elements such as riboswitches and other RNA biosensors provide an opportunity to detect molecules with high sensitivity. This is exemplified by the work in which engineered *S. cerevisiae* strains with enhanced tyrosine production were obtained using a RNA-aptamer-in-droplet (RAPID) system (Abatemarco et al., 2017).

In the second type of biosensors, producer strains engineered to produce high-yields of a chemical compound can be identified by co-cultivation with an auxotroph indicator strain. The sensor strain is auxotrophic for the desired compound and constitutively expresses a fluorescent protein (Fig. 6B). This can be illustrated in a study where a lysine auxotrophic *E. coli* strain was used to determine the total content of lysine in different feed ingredients (Chalova et al., 2007). Noteworthy, a computational analysis in *E. coli* predicted strategies to obtain auxotrophic-dependent biosensors for 53 metabolites (Tepper and Shlomi, 2011). These findings could potentially be used in microdroplet technologies.

In the third biosensor types, the presence of a compound is coupled to a fluorescence signal (Fig. 6C). One method involves enzymes, for instance in a previous study the translation of lactate production by cyanobacteria into a fluorescence signal (Hammar et al., 2015). This translation was performed by coupling NADH production via lactate dehydrogenase combined to a NADH-dependent conversion of a fluorogenic substrate. Moreover, when the compound of interest is an enzyme itself, the addition of fluorogenic substrates can couple the enzyme activity to fluorescence (Huebner et al., 2008; Bahls et al., 2017; Girault et al., 2018; Huang et al., 2018). Another method is based on growth inhibition of a sensor strain when the producer strain releases molecules with an inhibitory effect (e.g. antimicrobial compounds). The cell death can be detected by losing the cell fluorescence signal, by staining death cells, or by using a fluorogenic molecule. A study in *L. lactis* identified

variants of lanthipeptides with improved activity against pathogenic bacteria based on the principle of using micro compartments for antibiotic screening (Schmitt et al., 2019). The growth inhibition of constitutively-green fluorescent *Micrococcus flavus* cells (sensors) in these compartments indicated the production of an active peptide by *Lactococcus lactis* cells (antimicrobial producers). A similar approach to discover novel antimicrobials with a droplet platform identified the antibiotic amicoumacin A by cocultivation of oral microbiota of the Siberian bear with the target *Staphylococcus aureus* producing GFP (Terekhov et al., 2018).

One of the main problems with microbial biosensors is their selectivity. However, highly selective biosensors can be obtained either by genetic engineering methods or adapting the microorganism to the desired compound. In this context, carbohydrates are the most common target of microbial sensors, mainly lactose and glucose because of their significance in food industry (A.N., P.V., & T.A., 2012). Previous studies in *Lactococcus lactis* have yielded engineered strains

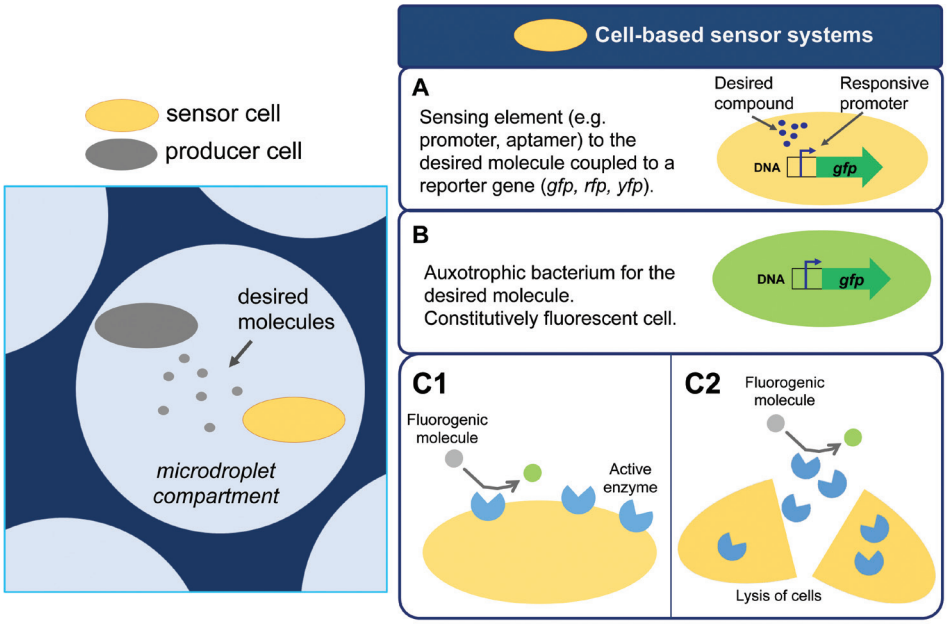

Figure 6. Cell-based sensors. The detection of a compound of interest by a biosensor using fluorescence can be achieved by different sensor systems: A-responsive promoters, B-auxotrophy for the compound of interest, C1-fluorescence via an enzymatic reaction and C2- coupling cell lysis to a fluorescence read out.

to grow in media containing a specific carbon source. By way of illustration, the disruption of the glucose uptake in *L. lactis* makes this bacterium suitable for lactose utilization (Pool et al., 2006). Since *L. lactis* is a good candidate in the production of carbohydrates and other nutraceuticals, these findings indicate the potential use of *L. lactis* strains as biosensors in microdroplets platforms.

From cell individuality to microbial communities

As single cells, bacteria have developed different mechanisms to accordingly respond to their environment. For instance, bacteria can change their morphology as a response to a change in the environment (Randich and Brun, 2015). Previous studies reveal that the shape diversity among bacteria is related to survival in diverse environments (Young, 2007). The bacterial cell shape is determined by the cell wall, and the peptidoglycan (PG) is the major component of the cell wall in both Gram-positive and Gram-negative bacteria. Gram-positive bacteria have a thick PG layer that is exposed to the external environment, including other components such as teichoic acids and mycolic acids (Margolin, 2009). The importance of the cell wall relies on the fact that this structure is the interface between the cell and its environment, and thus it contributes to the bacterial interactions with surfaces or other organisms (Yang et al., 2016; Vermassen et al., 2019).

Previous research on the cell wall disruption of LAB has been undertaken to favor the release of intracellular compounds (lysis) during cheese ripening and in this way to increase the organoleptic properties of food products (Chapot-Chartier et al., 1994; Crow et al., 1995). However, the PG remodeling is a tightly regulated process to prevent cell lysis. PG hydrolysis is required during the constant growth and separation of bacterial cells, but the presence of a strong PG structure defines the cell shape and protects it from the environment (Chapot-Chartier and Kulakauskas, 2014; Vermassen et al., 2019). In *L. lactis*, several enzymes participate in the PG hydrolysis. AcmA is the main *L. lactis* autolysin, which is degraded by extracellular proteinase PrtP (Buist et al., 1998). The autolysis of *L. lactis* MG1363 depends on the expression of the plasmid encoded cell wall-anchored proteinases PrtP-I and PrtP-III. Moreover, the cell wall polymers such as teichoic acids can modulate autolytic activity by shielding PG. For instance, in *L. lactis*, cell wall polymers and their modifications (e.g. glycosylation) hinder the binding of AcmA to PG (Steen et al., 2008).

Bacteria not only regulate the single cell processes such as the ones involved in cell division and peptidoglycan remodeling mentioned above, but also

they are able to regulate processes that concern the bacterial population. Environmental changes such as nutrient limitation trigger a bacterial response at the population level (Gasperotti et al., 2020). In this respect, diversity within the bacterial population benefit the bacterial members as a whole, for instance when a subpopulation of bacterial cell that survive by developing motility, spore formation or antimicrobial resistance (De Jong et al., 2011; Davis and Isberg, 2016).

Heterogeneity within an isogenic population is a common strategy that usually provides a selective advantage during an environmental change. Heterogeneity can originate from genetic variations (mutations), or non-genetic variations (phenotypic heterogeneity) (Grote et al., 2015). In general, there are two heterogeneity strategies that bacteria employ: bet-hedging or an environmental-driven strategy. The bet-hedging concept implies that the heterogeneity is maintained in the population and only some individual cells are able to survive when there is a change in the environment. In the environmental-driven strategy the environmental change triggers differential gene expression in some cells (Davis and Isberg, 2016). From an evolutionary point of view, it has been suggested that phenotypic diversification emerges from either molecular noise or as an evolved strategy (Li and Xie, 2011; Rivoire and Leibler, 2011). Phenotypic heterogeneity has been previously described in *L. lactis*, a bet-hedging strategy during diauxic shift. When the bacteria grow in media with two carbon sources, they consume first glucose, and subsequently only a subpopulation is able to utilize cellobiose (Solopova et al., 2014). This phenotypic individuality of bacteria is relevant in different fields of microbiology, from the case of persister cells with antimicrobial resistance, to the improvement in high-scale production of compounds with biotechnological or medical application.

Recently, a considerable literature has built up around the view of bacteria beyond their individuality and behavior in isogenic populations, as members of a microbial community composed of different bacterial species (Hibbing et al., 2010; D'Souza et al., 2018; Gonzalez et al., 2018). The LAB food fermentations are examples of the interactions that occur between members of a mixed-culture. The bacterial composition of a mixed-culture can directly influence the organoleptic properties of fermentation products, or affect the reproducibility of the fermentation process (Sieuwerts et al., 2008). One example of bacterial interactions in food fermentations is the commensalism between proteinase positive (PrtP+) and proteinase negative (PrtP-) *L. lactis* strains in the production of Gouda cheeses (Hugenholtz et al.,

1987). In this interaction, PrtP- cells are unable to breakdown casein, but survive by importing peptides released by PrtP+ cells. The yoghurt bacteria are another interesting example of bacterial interactions, where two thermophilic LAB, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* participate (Bolotin et al., 2004; Van De Guchte et al., 2006). Besides both bacteria produce lactic acid, several compounds are exchanged between them. *S. thermophilus* provides *L. delbrueckii* subsp. *bulgaricus* with formic acid, pyruvic acid, folic acid, whereas the proteolytic activity of *L. delbrueckii* subsp. *bulgaricus* releases casein-derived peptides that support the growth of *S. thermophilus* (Kingma, 1982; Crittenden et al., 2003).

The study of bacterial interactions might benefit several bacterial traits and their application. In fermentations, the understanding of competence, biofilm formation, exopolysaccharide (EPS) formation, and stress responses is utilized to improve the production of fermented foods (Rallu et al., 2000; Oliveira et al., 2015; Zengler and Zaramela, 2018). For example, enhanced production of the capsular EPS kefiran was performed by physical contact between *Lactobacillus kefiranofaciens* and *S. cerevisiae* (Cheirsilp et al., 2003). These physical and chemical interactions are widespread in many other environments, e.g. the common interactions between bacteria that inhabit our skin or guts.

This thesis

The work presented in this thesis mostly focuses on *L. lactis* and its role in the flavor formation, with special interest in the development of biosensors to detect compounds of interest. However, that initial research path took me to other research questions that I wanted to answer. Therefore, in essence, this thesis is divided in two different topics. The first part (**Chapters 2, 3, 4 & 5**) comprehends the development of *L. lactis* biosensors for the detection of compounds that directly or indirectly promote flavor formation in dairy fermentations. The second part is the result of my curiosity-driven research, and it comprehends different topics. Here, I show my interest on bacteria as single cells that perform essential processes such as cell division (**Chapter 6**), as single-species populations that import nutrients in a heterogeneous way (**Chapter 7**) and as members of a mixed-cell population that have to interact with other species (**Chapter 8**). Briefly, the content of each thesis chapter will be described below.

Part I. Development of bacterial biosensors to detect flavor-promoting compounds

Chapter 2 presents our work on the

development of *L. lactis* fluorescence-based biosensors for detection of the flavor compounds diacetyl and acetaldehyde. We performed transcriptome analyses to identify responsive promoters to diacetyl and acetaldehyde. Next, the candidate promoters were used to construct transcription-based sensors, i.e. a diacetyl or acetaldehyde responsive promoter driving the expression of GFP. We validated the functionality of the biosensors to respond to the presence of the compounds of interest by GFP expression. Moreover, we characterized the concentration range and cross-induction of the diacetyl-biosensors. Last, we applied the biosensors to correlate the diacetyl concentration in bacterial supernatants with the fluorescence signals from the biosensors. The biosensors developed in this study may eventually be used in the screening of LAB strains with increased diacetyl or acetaldehyde production, or in the detection of these compounds in complex food matrices.

Chapter 3 is an announcement of the genomic sequences of three *L. lactis* strains with amino acid secretion capacity. We studied the amino acid secretion capacity of *Lactococcus lactis* strains, and our screening revealed three amino acid-secreting strains: WW4, NCDO176 and C17. The availability of these data can be employed to identify the mechanisms involved in amino acid secretion by these bacteria. Factors that are expected to be associated with this property are either mutations in genes encoding nitrogen regulators, or the presence of genes encoding homologs of proteins that have been linked to amino acid secretion in other bacteria

Chapter 4 shows our work on the detection and selection of *L. lactis* strains with improved amino acid production and secretion. We developed a biosensing- and selection system that tackles the problem of selection for overproduction of secreted amino acids, by coupling the production of these molecules to the producing cell, i.e. in microdroplets that contain producer and biosensor bacteria. We constructed a growth-based sensor strain to detect the amino acids isoleucine, leucine, valine, histidine and methionine. Amino acid biosensors can simplify the quantification of free amino acids in complex (food) matrices, but also can facilitate the screening process of producers by their combination with high-throughput approaches. With regard to the latter, we used *Lactococcus lactis* strains to benchmark the performance of the amino acid biosensor, and identified wild-type strains with high-yield amino acid secretion. Subsequently, we used this biosensor in combination with a droplet-based screening approach, and isolated three mutated *L. lactis* IPLA838 strains with 5-10 fold increased amino

acid-secretion capacity compared to the wild type. Genome re-sequencing revealed mutations in genes encoding proteins that participate in peptide uptake and peptide degradation.

Chapter 5 presents our work on the development of *L. lactis* biosensors for detection of the sulfur-containing amino acids, methionine and cysteine. We employed two strategies to create these biosensors, the first one is based on the methionine auxotrophy of this bacterium and the second strategy is based on a cysteine-responsive promoter. The characterization of the biosensors confirms their response to the presence of these amino acids. The biosensors developed in this study may eventually be used to engineer strains or pathways for increased methionine and cysteine production, and may facilitate the detection of these amino acids in food matrices.

Part II. A view of bacteria at the single-cell, single-species culture, and mixed-species level

Chapter 6 shows our work on the view of bacteria at the single-cell level. Usp45 is the most highly expressed secreted protein of *L. lactis*, but its biological function remained neglected for more than 25 years. Thus, we aimed to shed light on the role of Usp45 in *L. lactis*. We assessed the essentiality of the *usp45* gene in *L. lactis*, and failed to obtain deletion mutants. We tackled this limitation by knocking-down *usp45* by using a CRISPR interference method. A growth defect and aberrant cell shape formation resulted from the repression of transcription of *usp45*. Our findings suggest that Usp45 is an essential peptidoglycan hydrolase for proper cell division, a role that is in agreement with the findings obtained in other studies on Usp45 homologs in other Gram-positive bacteria. Moreover, by analyzing the growth conditions that trigger the activation of the *usp45* promoter, we discovered that this promoter is highly activated by galactose. Given that we observed the same galactose effect on the promoter of *acmA*, encoding the major autolysin in *L. lactis*, we speculate that galactose affects the Usp45 activity in a similar way as reported previously for AcmA.

Chapter 7 uncovers a remarkable case of long-term phenotypic heterogeneity in *L. lactis*, in a single-species population. When methionine becomes limiting, two isogenic subpopulations emerge that rely on different methionine transporters to support growth: one subpopulation mostly relies on the high-affinity transporter and another subpopulation on the low-affinity transporter. The phenotypic heterogeneity is incredibly stable and inherited for tens of generations, making heterogeneity even apparent at the colony

level. We analyze nearly 10,000 of these colonies using automatic image analysis and subsequently show that the long-term phenotypic heterogeneity results from a T-box riboswitch in the promoter region of the high-affinity transporter. To our knowledge, this is the first case of RNA-level regulation that gives rise to phenotypic heterogeneity. Given that T-box regulation is commonly found in auxotrophic bacteria, like those inhabiting our guts, we speculate that long-term phenotypic heterogeneity in amino acid uptake might be widespread.

Chapter 8 is a study about the social behavior of bacteria such as the interaction of bacterial species in a mixed-species population. We aimed to study the interactions that bacteria are able to establish in a densely populated environment. Thus, we study the interactions between two members of our skin microbiota, *Bacillus subtilis* and *Staphylococcus epidermidis*. We discovered that *B. subtilis* actively responds to the presence of *S. epidermidis* in the proximity, by two strategies: antimicrobial production and development of a subpopulation with migratory response. The initial response of *B. subtilis* is production of chlorotetain to partially degrade *S. epidermidis* at the colony level. Next, a subpopulation of *B. subtilis* motile cells emerges. Remarkably this subpopulation slides towards the remaining *S. epidermidis* colony and engulfs it. We hypothesized that this attack and back down response from *B. subtilis* and *S. epidermidis* respectively, which resembles other conflicts in nature, as the ones in animals, may play a role in defining the bacterial species and the specific microenvironments that these bacteria occupy in or on our skin.

In **Chapter 9,** I summarize the most important findings of the research described in this thesis. This work advances our knowledge of the *L. lactis* metabolism, physiology and biotechnological potential. In addition, I discuss the development of high genetic diversity in the *L. lactis* species through the process of domestication and some unanswered questions, which would be a fruitful area for future research.

REFERENCES

Abatemarco, J., Sarhan, M. F., Wagner, J. M., Lin, J. L., Liu, L., Hassouneh, W., et al. (2017). RNA-aptamers-in-droplets (RAPID) high-throughput screening for secretory phenotypes. *Nat. Commun.* doi:10.1038/s41467-017-00425-7.

Aller, K., Adamberg, K., Timarova, V., Seiman, A., Feštšenko, D., and Vilu, R. (2014). Nutritional requirements and media development for *Lactococcus lactis* IL1403. *Appl. Microbiol. Biotechnol.* doi:10.1007/s00253-014-5641-7.

Arakawa, T., Tsumoto, K., Kita, Y., Chang, B., and Ejima, D. (2007). Biotechnology applications of amino acids in protein purification and formulations. *Amino Acids.* doi:10.1007/s00726-007-0506-3.

Bahls, M. O., Kardashliev, T., and Panke, S. (2017). "Novel sensors for engineering microbiology," in *Consequences of microbial interactions with hydrocarbons, oils, and lipids: production of fuels and chemicals*, ed. S. Y. Lee (Springer), 331–357. doi:10.1007/978-3-319-50436-0.

Benson, K. H., Godon, J. J., Renault, P., Griffin, H. G., and Gasson, M. J. (1996). Effect of *ilvBN*-encoded α-acetolactate synthase expression on diacetyl production in *Lactococcus lactis. Appl. Microbiol. Biotechnol.* doi:10.1007/s002530050656.

Bolotin, A., Quinquis, B., Renault, P., Sorokin, A., Ehrlich, S. D., Kulakauskas, S., et al. (2004). Complete sequence and comparative genome analysis of the dairy bacterium *Streptococcus thermophilus. Nat. Biotechnol.* doi:10.1038/nbt1034.

Bongaerts, J., Krämer, M., Müller, U., Raeven, L., and Wubbolts, M. (2001). Metabolic engineering for microbial production of aromatic amino acids and derived compounds. *Metab. Eng.* doi:10.1006/mben.2001.0196.

Børsting, M. W., Qvist, K. B., Brockmann, E., Vindeløv, J., Pedersen, T. L., Vogensen, F. K., et al. (2015). Classification of *Lactococcus lactis* cell envelope proteinase based on gene sequencing, peptides formed after hydrolysis of milk, and computer modeling. *J. Dairy Sci.* doi:10.3168/jds.2014-8517.

Buist, G., Venema, G., and Kok, J. (1998). Autolysis of *Lactococcus lactis* is influenced by proteolysis. *J. Bacteriol.* doi:10.1128/jb.180.22.5947-5953.1998.

Cavanagh, D., Fitzgerald, G. F., and McAuliffe, O. (2015). From field to fermentation: The origins of *Lactococcus lactis* and its domestication to the dairy environment. *Food Microbiol.* doi:10.1016/j.fm.2014.11.001.

Chalova, V. I., Kim, W. K., Woodward, C. L., and Ricke, S. C. (2007). Quantification of total and bioavailable lysine in feed protein sources by a whole-cell green fluorescent protein growth-based *Escherichia coli* biosensor. *Appl. Microbiol. Biotechnol.* doi:10.1007/s00253-007-0989-6.

Chambellon, E., and Yvon, M. (2003). CodY-regulated aminotransferases AraT and BcaT play a major role in the growth of *Lactococcus lactis* in milk by regulating the intracellular pool of amino acids. *Appl. Environ. Microbiol.* doi:10.1128/AEM.69.6.3061-3068.2003.

Chapot-Chartier, M. P., Deniel, C., Rousseau, M., Vassal, L., and Gripon, J. C. (1994). Autolysis of two strains of *Lactococcus lactis* during cheese ripening. *Int. Dairy J.* doi:10.1016/0958-6946(94)90016-7.

Chapot-Chartier, M. P., and Kulakauskas, S. (2014). Cell wall structure and function in lactic acid bacteria. *Microb. Cell Fact.* doi:10.1186/1475-2859-13-S1-S9.

Cheirsilp, B., Shoji, H., Shimizu, H., and Shioya, S. (2003). Interactions between *Lactobacillus kefiranofacien*s and *Saccharomyces*

cerevisiae in Mixed Culture for Kefiran Production. *J. Biosci. Bioeng.* doi:10.1016/S1389-1723(03)80194-9.

Christensen, J. E., Dudley, E. G., Pederson, J. A., and Steele, J. L. (1999). Peptidases and amino acid catabolism in lactic acid bacteria. in *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology* doi:10.1023/A:1002001919720.

Clark, S., and Winter, C. K. (2015). Diacetyl in Foods: A Review of Safety and Sensory Characteristics. *Compr. Rev. Food Sci. Food Saf.* doi:10.1111/1541-4337.12150.

Crittenden, R. G., Martinez, N. R., and Playne, M. J. (2003). Synthesis and utilisation of folate by yoghurt starter cultures and probiotic bacteria. *Int. J. Food Microbiol.* doi:10.1016/S0168-1605(02)00170-8.

Crow, V. L., Martley, F. G., Coolbear, T., and Roundhill, S. J. (1995). The influence of phage-assisted lysis of *Lactococcus lactis* subsp. *lactis* ML8 on cheddar cheese ripening. *Int. Dairy J.* doi:10.1016/0958-6946(95)00022-U.

D'Este, M., Alvarado-Morales, M., and Angelidaki, I. (2018). Amino acids production focusing on fermentation technologies – A review. *Biotechnol. Adv.* doi:10.1016/j.biotechadv.2017.09.001.

D'Souza, G., Shitut, S., Preussger, D., Yousif, G., Waschina, S., and Kost, C. (2018). Ecology and evolution of metabolic cross-feeding interactions in bacteria. *Nat. Prod. Rep.* doi:10.1039/c8np00009c.

Davis, K. M., and Isberg, R. R. (2016). Defining heterogeneity within bacterial populations via single cell approaches. *BioEssays.* doi:10.1002/bies.201500121.

de Jong, I. G., Haccou, P., and Kuipers, O. P. (2011). Bet hedging or not? A guide to proper classification of microbial survival strategies. *BioEssays.* doi:10.1002/bies.201000127.

den Hengst, C. D., Groeneveld, M., Kuipers, O. P., and Kok, J. (2006). Identification and functional characterization of the *Lactococcus lactis* CodY-regulated branched-chain amino acid permease BcaP (CtrA). *J. Bacteriol.* 188, 3280–3289. doi:10.1128/JB.188.9.3280-3289.2006.

den Hengst, C. D., van Hijum, S. A. F. T., Geurts, J. M. W., Nauta, A., Kok, J., and Kuipers, O. P. (2005). The *Lactococcus lactis* CodY Regulon . *J. Biol. Chem.* doi:10.1074/jbc.m502349200.

Drake, S. L., Carunchia Whetstine, M. E., Drake, M. A., Courtney, P., Fligner, K., Jenkins, J., et al. (2007). Sources of umami taste in Cheddar and Swiss cheeses. *J. Food Sci.* doi:10.1111/j.1750-3841.2007.00402.x.

Exterkate, F. A., Alting, A. C., and Bruinenberg, P. G. (1993). Diversity of cell envelope proteinase specificity among strains of *Lactococcus lactis* and its relationship to charge characteristics of the substrate-binding region. *Appl. Environ. Microbiol.* 59, 3640–3647. doi:10.1128/aem.00247-08.

Flahaut, N. A. L., Wiersma, A., Van De Bunt, B., Martens, D. E., Schaap, P. J., Sijtsma, L., et al. (2013). Genome-scale metabolic model for *Lactococcus lactis* MG1363 and its application to the analysis of flavor formation. *Appl. Microbiol. Biotechnol.* doi:10.1007/s00253-013-5140-2.

Gasperotti, A., Brameyer, S., Fabiani, F., and Jung, K. (2020). Phenotypic heterogeneity of microbial populations under nutrient limitation. *Curr. Opin. Biotechnol.* doi:10.1016/j.copbio.2019.09.016.

Georgi, T., Rittmann, D., and Wendisch, V. F. (2005). Lysine and glutamate production by *Corynebacterium glutamicum* on glucose, fructose and sucrose: Roles of malic enzyme and fructose-1,6-bisphosphatase. *Metab. Eng.* doi:10.1016/j.ymben.2005.05.001.

Girault, M., Beneyton, T., Pekin, D., Buisson, L., Bichon, S., Charbonniers, C., et al. (2018). High-content screening of plankton alkaline phosphatase activity in microfluidics. *analy* 90, 4174–4181. doi:10.1021/acs.analchem.8b00234.

Gonzalez, D., Sabnis, A., Foster, K. R., and Mavridou, D. A. I. (2018). Costs and benefits of provocation in bacterial warfare. *Proc. Natl. Acad. Sci. U. S. A.* doi:10.1073/pnas.1801028115.

Green, N. J., Grundy, F. J., and Henkin, T. M. (2010). The T box mechanism: tRNA as a regulatory molecule. *FEBS Lett.* doi:10.1016/j.febslet.2009.11.056.

Grote, J., Krysciak, D., and Streit, W. R. (2015). Phenotypic heterogeneity, a phenomenon that may explain why quorum sensing does not always result in truly homogenous cell behavior. *Appl. Environ. Microbiol.* doi:10.1128/AEM.00900-15.

Guédon, E., Serror, P., Ehrlich, S. D., Renault, P., and Delorme, C. (2001). Pleiotropic transcriptional repressor CodY senses the intracellular pool of branched-chain amino acids in *Lactococcus lactis*. *Mol. Microbiol.* 40, 1227–1239. doi:10.1046/j.1365-2958.2001.02470.x.

Gutiérrez-Méndez, N., Vallejo-Cordoba, B., González-Córdova, A. F., Nevárez-Moorillón, G. V., and Rivera-Chavira, B. (2008). Evaluation of aroma generation of *Lactococcus lactis* with an electronic nose and sensory analysis. *J. Dairy Sci.* doi:10.3168/jds.2007-0193.

Gutierrez-Preciado, A., Henkin, T. M., Grundy, F. J., Yanofsky, C., and Merino, E. (2009). Biochemical Features and Functional Implications of the RNA-Based T-Box Regulatory Mechanism. *Microbiol. Mol. Biol. Rev.* doi:10.1128/mmbr.00026-08.

Haandrikman, A. J., Kok, J., Laan, H., Soemitro, S., Ledeboer, A. M., Konings, W. N., et al. (1989). Identification of a gene required for maturation of an extracellular lactococcal serine proteinase. *J. Bacteriol.* doi:10.1128/jb.171.5.2789-2794.1989.

Haandrikman, A. J., Meesters, R., Laan, H., Konings, W. N., Kok, J., and Venema, G. (1991). Processing of the lactococcal extracellular serine proteinase. *Appl. Environ. Microbiol.* doi:10.1128/aem.57.7.1899-1904.1991.

Hagting, A., Kunji, E. R. S., Leenhouts, K. J., Poolman, B., and Konings, W. N. (1994). The Di- and tripeptide transport protein of *Lactococcus lactis*. A new type of bacterial peptide transporter. *J. Biol. Chem.*

Hammar, P., Angermayr, S. A., Sjostrom, S. L., Van Der Meer, J., Hellingwerf, K. J., Hudson, E. P., et al. (2015). Single-cell screening of photosynthetic growth and lactate production by cyanobacteria. *Biotechnol. Biofuels*. doi:10.1186/s13068-015-0380-2.

Henkin, T. M., and Grundy, F. J. (2006). Sensing metabolic signals with nascent RNA tanscripts: The T box and S box riboswitches as paradigms. in *Cold Spring Harbor Symposia on Quantitative Biology* doi:10.1101/sqb.2006.71.020.

Hibbing, M. E., Fuqua, C., Parsek, M. R., and Peterson, S. B. (2010). Bacterial competition: Surviving and thriving in the microbial jungle. *Nat. Rev. Microbiol.* doi:10.1038/nrmicro2259.

Hirasawa, T., and Shimizu, H. (2016). Recent advances in amino acid production by microbial cells. *Curr. Opin. Biotechnol.* doi:10.1016/j.copbio.2016.04.017.

Huang, M., Joensson, H. N., and Nielsen, J. (2018). "High-throughput microfluidics for the screening of yeast libraries," in *Synthetic metabolic pathways: methods and protocols*, eds. M. K. Jensen and J. D. Keasling (Springer), 307–317.

Huebner, A., Olguin, L. F., Bratton, D., Whyte, G., Huck, W. T. S., De Mello, A. J., et al. (2008). Development of quantitative cell-based enzyme assays in microdroplets. *Anal. Chem.* doi:10.1021/ac800338z.

Hugenholtz, J., Kleerebezem, M., Starrenburg, M., Delcour, J., De Vos, W., and Hols, P. (2000). *Lactococcus lactis* as a cell factory for high-level diacetyl production. *Appl. Environ. Microbiol.* 66, 4112–4114. doi:10.1128/AEM.66.9.4112-4114.2000.

Hugenholtz, J., Splint, R., Konings, W. N., and Veldkamp, H. (1987). Selection of protease-positive and protease-negative variants of *Streptococcus cremoris. Appl. Environ. Microbiol.* doi:10.1128/aem.53.2.309-314.1987.

Ikeda, M. (2003). Amino acid production processes. *Adv. Biochem. Eng. Biotechnol.* doi:10.1007/3-540-45989-8_1.

Juillard, V., Laan, H., Kunji, E. R. S., Jeronimus-Stratingh, C. M., Bruins, A. P., and Konings, W. N. (1995). The extracellular P(I)-type proteinase of *Lactococcus lactis* hydrolyzes β-casein into more than one hundred different oligopeptides. *J. Bacteriol.* 177, 3472–3478.

Karimi, R., Sohrabvandi, S., and Mortazavian, A. M. (2012). Review Article: Sensory Characteristics of Probiotic Cheese. *Compr. Rev. Food Sci. Food Saf.* doi:10.1111/j.1541-4337.2012.00194.x.

Kieronczyk, A., Skeie, S., Langsrud, T., Le Bars, D., and Yvon, M. (2004). The nature of aroma compounds produced in a cheese model by glutamate dehydrogenase positive *Lactobacillus* INF15D depends on its relative aminotransferase activities towards the different amino acids. *Int. Dairy J.* doi:10.1016/j.idairyj.2003.07.001.

Kieronczyk, A., Skeie, S., Langsrud, T., and Yvon, M. (2003). Cooperation between *Lactococcus lactis* and nonstarter lactobacilli in the formation of cheese aroma from amino acids. *Appl. Environ. Microbiol.* doi:10.1128/AEM.69.2.734-739.2003.

Kingma, F. (1982). Evidence that *Lactobacillus bulgaricus* in yogurt is stimulated by carbon dioxide produced by Streptococcus thermophilus. *Netherlands Milk Dairy J.*

Kleerebezem, M., Hols, P., and Hugenholtz, J. (2000). Lactic acid bacteria as a cell factory: Rerouting of carbon metabolism in *Lactococcus lactis* by metabolic engineering. *Enzyme Microb. Technol.* 26, 840–848. doi:10.1016/S0141-0229(00)00180-0.

Kok, J. (1990). Genetics of the proteolytic system of lactic acid bacteria. *FEMS Microbiol. Lett.* doi:10.1016/0378-1097(90)90695-M.

Krämer, R. (1994). Secretion of amino acids by bacteria: Physiology and mechanism. *FEMS Microbiol. Rev.* doi:10.1016/0168-6445(94)90102-3.

Kunji, E. R. S., Fang, G., Jeronimus-Stratingh, C. M., Bruins, A. P., Poolman, B., and Konings, W. N. (1998). Reconstruction of the proteolytic pathway for use of β-casein by *Lactococcus lactis*. *Mol. Microbiol.* doi:10.1046/j.1365-2958.1998.00769.x.

Kunji, E. R. S., Hagting, A., De Vries, C. J., Juillard, V., Haandrikman, A. J., Poolman, B., et al. (1995). Transport of β-casein-derived peptides by the oligopeptide transport system is a crucial step in the proteolytic pathway of *Lactococcus lactis*. *J. Biol. Chem.* doi:10.1074/jbc.270.4.1569.

Kunji, E. R. S., Smid, E. J., Plapp, R., Poolman, B., and Konings, W. N. (1993). Di-tripeptides and oligopeptides are taken up via distinct transport mechanisms in *Lactococcus lactis. J. Bacteriol.* doi:10.1128/jb.175.7.2052-2059.1993.

Laan, H., Bolhuis, H., Poolman, B., Abee, T., and Konings, W. N. (1993). Regulation of proteinase synthesis in *Lactococcus lactis. Acta Biotechnol.* doi:10.1002/abio.370130202.

Lepore, B. W., Indic, M., Pham, H., Hearn, E. M., Patel, D. R., and Van Den Berg, B. (2011). Ligand-gated diffusion across the bacterial outer membrane. *Proc. Natl. Acad. Sci. U. S. A.* doi:10.1073/pnas.1018532108.

Li, G. W., and Xie, X. S. (2011). Central dogma at the single-molecule level in living cells. *Nature.* doi:10.1038/nature10315.

Li, Y., Ogola, H. J. O., and Sawa, Y. (2012). L-Aspartate dehydrogenase: Features and applications. *Appl. Microbiol. Biotechnol.* doi:10.1007/s00253-011-3730-4.

Lim, J. W., Ha, D., Lee, J., Lee, S. K., and Kim, T. (2015). Review of micro/nanotechnologies for microbial biosensors. *Front. Bioeng. Biotechnol.* doi:10.3389/fbioe.2015.00061.

Liu, M., Prakash, C., Nauta, A., Siezen, R. J., and Francke, C. (2012). Computational analysis of cysteine and methionine metabolism and its regulation in dairy starter and related bacteria. *J. Bacteriol.* doi:10.1128/JB.06816-11.

Mahr, R., and Frunzke, J. (2016). Transcription factor-based biosensors in biotechnology: current state and future prospects. *Appl. Microbiol. Biotechnol.* doi:10.1007/s00253-015-7090-3.

Margolin, W. (2009). Sculpting the Bacterial Cell. *Curr. Biol.* doi:10.1016/j.cub.2009.06.033.

Marin, K., and Krämer, R. (2007). "Amino Acid Transport Systems in Biotechnologically Relevant Bacteria," in *Amino Acid Biosynthesis ~ Pathways, Regulation and Metabolic Engineering* doi:10.1007/7171_2006_069.

Marreddy, R. K. R., Geertsma, E. R., Permentier, H. P., Pinto, J. P. C., Kok, J., and Poolman, B. (2010). Amino acid accumulation limits the overexpression of proteins in *Lactococcus lactis.*

PLoS One. doi:10.1371/journal.pone.0010317.

McAuliffe, O. (2018). Symposium review: *Lactococcus lactis* from nondairy sources: Their genetic and metabolic diversity and potential applications in cheese1. *J. Dairy Sci.* doi:10.3168/jds.2017-13331.

Meijer, W., Marugg, J. D., and Hugenholtz, J. (1996). Regulation of proteolytic enzyme activity in *Lactococcus lactis. Appl. Environ. Microbiol.* doi:10.1128/aem.62.1.156-161.1996.

Mitsuhashi, S. (2014). Current topics in the biotechnological production of essential amino acids, functional amino acids, and dipeptides. *Curr. Opin. Biotechnol.* doi:10.1016/j.copbio.2013.08.020.

Morello, E., Bermúdez-Humarán, L. G., Llull, D., Solé, V., Miraglio, N., Langella, P., et al. (2007). *Lactococcus lactis,* an efficient cell factory for recombinant protein production and secretion. *J. Mol. Microbiol. Biotechnol.* doi:10.1159/000106082.

Neves, A. R., Pool, W. A., Kok, J., Kuipers, O. P., and Santos, H. (2005). Overview on sugar metabolism and its control in *Lactococcus lactis* - The input from in vivo NMR. *FEMS Microbiol. Rev.* doi:10.1016/j.femsre.2005.04.005.

Nikaido, H. (1993). Transport across the bacterial outer membrane. *J. Bioenerg. Biomembr.* doi:10.1007/BF00770245.

Niven, G. W., Knight, D. J., and Mulholland, F. (1998). Changes in the concentrations of free amino acids in milk during growth of *Lactococcus lactis* indicate biphasic nitrogen metabolism. *J. Dairy Res.* doi:10.1017/S002202999700263X.

Oliveira, N. M., Martinez-Garcia, E., Xavier, J., Durham, W. M., Kolter, R., Kim, W., et al. (2015). Biofilm formation as a response to ecological competition. *PLoS Biol.* doi:10.1371/journal.pbio.1002191.

Parapouli, M., Delbès-Paus, C., Kakouri, A., Koukkou, A. I., Montel, M. C., and Samelis, J. (2013). Characterization of a wild, novel nisin A-Producing *Lactococcus* strain with an *L. lactis* subsp. *cremoris* genotype and an *L. lactis* subsp. lactis *phenotype*, isolated from Greek raw milk. *Appl. Environ. Microbiol.*

doi:10.1128/AEM.00436-13.

Passerini, D., Beltramo, C., Coddeville, M., Quentin, Y., Ritzenthaler, P., Daveran-Mingot, M. L., et al. (2010). Genes but Not Genomes Reveal Bacterial Domestication of *Lactococcus Lactis*. *PLoS One*. doi:10.1371/journal.pone.0015306.

Pinu, F. R., Granucci, N., Daniell, J., Han, T. L., Carneiro, S., Rocha, I., et al. (2018). Metabolite secretion in microorganisms: the theory of metabolic overflow put to the test. *Metabolomics*. doi:10.1007/s11306-018-1339-7.

Ponomarova, O., Gabrielli, N., Sévin, D. C., Mülleder, M., Zirngibl, K., Bulyha, K., et al. (2017). Yeast Creates a Niche for Symbiotic Lactic Acid Bacteria through Nitrogen Overflow. *Cell Syst.* doi:10.1016/j.cels.2017.09.002.

Pool, W. A., Neves, A. R., Kok, J., Santos, H., and Kuipers, O. P. (2006). Natural sweetening of food products by engineering *Lactococcus lactis* for glucose production. *Metab. Eng.* doi:10.1016/j.ymben.2006.05.003.

Quintans, N. G., Blancato, V., Repizo, G., Magni, C., and Lopez, P. (2008). Citrate metabolism and aroma compound production in lactic acid bacteria. *Mol. Asp. Lact. Acid Bact. Tradit. New Appl.* doi:978-81-308-0250-3.

Rallu, F., Gruss, A., Ehrlich, S. D., and Maguin, E. (2000). Acid- and multistress-resistant mutants of *Lactococcus lactis*: Identification of intracellular stress signals. *Mol. Microbiol.* doi:10.1046/j.1365-2958.2000.01711.x.

Randich, A. M., and Brun, Y. V. (2015). Molecular mechanisms for the evolution of bacterial morphologies and growth modes. *Front. Microbiol.* doi:10.3389/fmicb.2015.00580.

Rivoire, O., and Leibler, S. (2011). The Value of Information for Populations in Varying Environments. *J. Stat. Phys.* doi:10.1007/s10955-011-0166-2.

Samaržija, D., Antunac, N., and Havranek, J. (2001). Taxonomy, physiology and growth of *Lactococcus lactis*: a review. *Mljekarstvo* 51, 35–48. Available at: http://hrcak.srce.hr/index.php?show=clanak_download&id_clanak_jezik=2828.

Sanz, Y., Lanfermeijer, F. C., Renault, P., Bolotin, A., Konings, W. N., and Poolman, B. (2001). Genetic and functional characterization of dpp genes encoding a dipeptide transport system in *Lactococcus lactis*. *Arch. Microbiol.* doi:10.1007/s002030100270.

Savijoki, K., Ingmer, H., and Varmanen, P. (2006). Proteolytic systems of lactic acid bacteria. *Appl. Microbiol. Biotechnol.* doi:10.1007/s00253-006-0427-1.

Schmitt, S., Montalbán-López, M., Peterhoff, D., Deng, J., Wagner, R., Held, M., et al. (2019). Analysis of modular bioengineered antimicrobial lanthipeptides at nanoliter scale. *Nat. Chem. Biol.* doi:10.1038/s41589-019-0250-5.

Schuurman-Wolters, G. K., and Poolman, B. (2005). Substrate specificity and ionic regulation of GlnPQ from *Lactococcus lactis*: An ATP-binding cassette transporter with four extracytoplasmic substrate-binding domains. *J. Biol. Chem.* doi:10.1074/jbc.M500522200.

Seefeldt, K. E., and Weimer, B. C. (2000). Diversity of sulfur compound production in lactic acid bacteria. *J. Dairy Sci.* doi:10.3168/jds.S0022-0302(00)75168-X.

Sieuwerts, S., De Bok, F. A. M., Hugenholtz, J., and Van Hylckama Vlieg, J. E. T. (2008). Unraveling microbial interactions in food fermentations: From classical to genomics approaches. *Appl. Environ. Microbiol.* doi:10.1128/AEM.00113-08.

Smit, B. A., Engels, W. J. M., and Smit, G. (2009). Branched chain aldehydes: Production and breakdown pathways and relevance for flavour in foods. *Appl. Microbiol. Biotechnol.* doi:10.1007/s00253-008-1758-x.

Solms, J. (1969). The Taste of Amino Acids, Peptides, and Proteins. *J. Agric. Food Chem.* doi:10.1021/jf60164a016.

Solopova, A., van Gestel, J., Weissing, F. J., Bachmann, H., Teusink, B., Kok, J., et al. (2014). Bet-hedging during bacterial diauxic shift. *Proc. Natl. Acad. Sci.* doi:10.1073/pnas.1320063111.

Song, A. A. L., In, L. L. A., Lim, S. H. E., and Rahim, R. A. (2017). A review on *Lactococcus lactis:* From food to factory. *Microb.*

Cell Fact. doi:10.1186/s12934-017-0669-x.

Sperandio, B., Polard, P., Ehrlich, D. S., Renault, P., and Guédon, E. (2005). Sulfur amino acid metabolism and its control in *Lactococcus lactis* IL1403. *J. Bacteriol.* doi:10.1128/JB.187.11.3762-3778.2005.

Steen, A., Buist, G., Kramer, N. E., Jalving, R., Benus, G. F. J. D., Venema, G., et al. (2008). Reduced lysis upon growth of *Lactococcus lactis* on galactose is a consequence of decreased binding of the autolysin AcmA. in *Applied and Environmental Microbiology* doi:10.1128/AEM.00103-08.

Suddala, K. C., Cabello-Villegas, J., Michnicka, M., Marshall, C., Nikonowicz, E. P., and Walter, N. G. (2018). Hierarchical mechanism of amino acid sensing by the T-box riboswitch. *Nat. Commun.* doi:10.1038/s41467-018-04305-6.

Tepper, N., and Shlomi, T. (2011). Computational design of Auxotrophy-dependent microbial biosensors for combinatorial metabolic engineering experiments. *PLoS One.* doi:10.1371/journal.pone.0016274.

Terekhov, S. S., Smirnov, I. V., Malakhova, M. V., Samoilov, A. E., Manolov, A. I., Nazarov, A. S., et al. (2018). Ultrahigh-throughput functional profiling of microbiota communities. *Proc. Natl. Acad. Sci.* doi:10.1073/pnas.1811250115.

Thakur, M. S., and Ragavan, K. V. (2013). Biosensors in food processing. *J. Food Sci. Technol.* doi:10.1007/s13197-012-0783-z.

Theberge, A. B., Courtois, F., Schaerli, Y., Fischlechner, M., Abell, C., Hollfelder, F., et al. (2010). Microdroplets in microfluidics: An evolving platform for discoveries in chemistry and biology. *Angew. Chemie - Int. Ed.* doi:10.1002/anie.200906653.

Thomas, T. D., and Pritchard, G. G. (1987). Proteolytic enzymes of dairy starter cultures. *FEMS Microbiol. Lett.* doi:10.1016/0378-1097(87)90111-X.

Trip, H., Mulder, N. L., and Lolkema, J. S. (2013). Cloning, expression, and functional characterization of secondary amino acid transporters of *Lactococcus lactis*. *J. Bacteriol.* doi:10.1128/JB.01948-12.

Tripathy, D. B., Mishra, A., Clark, J., and Farmer, T. (2018). Synthesis, chemistry, physicochemical properties and industrial applications of amino acid surfactants: A review. *Comptes Rendus Chim.* doi:10.1016/j.crci.2017.11.005.

van De Guchte, M., Penaud, S., Grimaldi, C., Barbe, V., Bryson, K., Nicolas, P., et al. (2006). The complete genome sequence of *Lactobacillus bulgaricus* reveals extensive and ongoing reductive evolution. *Proc. Natl. Acad. Sci. U. S. A.* doi:10.1073/pnas.0603024103.

van Kranenburg, R., Kleerebezem, M., Van Hylckama Vlieg, J., Ursing, B. M., Boekhorst, J., Smit, B. A., et al. (2002). Flavour formation from amino acids by lactic acid bacteria: Predictions from genome sequence analysis. *Int. Dairy J.* doi:10.1016/S0958-6946(01)00132-7.

van Niel, E. W. J., and Hahn-Hägerdal, B. (1999). Nutrient requirements of lactococci in defined growth media. *Appl. Microbiol. Biotechnol.* doi:10.1007/s002530051569.

van Tatenhove-Pel, R. J., Hernandez-Valdes, J. A., Teusink, B., Kuipers, O. P., Fischlechner, M., and Bachmann, H. (2020). Microdroplet screening and selection for improved microbial production of extracellular compounds. *Curr. Opin. Biotechnol.* doi:10.1016/j.copbio.2019.10.007.

Velasco, I., Tenreiro, S., Calderon, I. L., and André, B. (2004). Saccharomyces cerevisiae Aqr1 is an internal-membrane transporter involved in excretion of amino acids. *Eukaryot. Cell.* doi:10.1128/EC.3.6.1492-1503.2004.

Vermassen, A., Leroy, S., Talon, R., Provot, C., Popowska, M., and Desvaux, M. (2019). Cell wall hydrolases in bacteria: Insight on the diversity of cell wall amidases, glycosidases and peptidases toward peptidoglycan. *Front. Microbiol.* doi:10.3389/fmicb.2019.00331.

Wels, M., Kormelink, T. G., Kleerebezem, M., Siezen, R. J., and Francke, C. (2008). An in silico analysis of T-box regulated genes and T-box evolution in prokaryotes, with emphasis on prediction of substrate specificity of transporters. *BMC*

Genomics. doi:10.1186/1471-2164-9-330.

Wendisch, V. F. (2014). Microbial production of amino acids and derived chemicals: Synthetic biology approaches to strain development. *Curr. Opin. Biotechnol.* doi:10.1016/j.copbio.2014.05.004.

Yamaguchi, S., and Ninomiya, K. (2000). Umami and Food Palatability. *J. Nutr.* doi:10.1093/jn/130.4.921s.

Yang, D. C., Blair, K. M., and Salama, N. R. (2016). Staying in Shape: the Impact of Cell Shape on Bacterial Survival in Diverse Environments. *Microbiol. Mol. Biol. Rev.* doi:10.1128/mmbr.00031-15.

Young, K. D. (2007). Bacterial morphology: why have different shapes? *Curr. Opin. Microbiol.* doi:10.1016/j.mib.2007.09.009.

Yvon, M., and Rijnen, L. (2001). Cheese flavour formation by amino acid catabolism. in *International Dairy Journal* doi:10.1016/S0958-6946(01)00049-8.

Zahoor, A., Lindner, S. N., and Wendisch, V. F. (2012). Metabolic engineering of Corynebacterium glutamicum aimed at alternative carbon sources and new products. *Comput. Struct. Biotechnol. J.* doi:10.5936/csbj.201210004.

Zengler, K., and Zaramela, L. S. (2018). The social network of microorganisms - How auxotrophies shape complex communities. *Nat. Rev. Microbiol.* doi:10.1038/s41579-018-0004-5.

PART I

Development of bacterial biosensors to detect flavor-promoting compounds